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Regulation of TIMP-1 phenotypic expression in Epstein–Barr virus-immortalized B lymphocytes

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Abstract

Normal B lymphocytes as well as malignant B cells extravasate from blood circulation during physiological and pathological processes and require matrix metalloproteinases (MMPs) to facilitate trafficking through the subendothelial basal lamina and the extracellular matrix. We have previously shown that Epstein–Barr virus (EBV)-immortalized B lymphocytes constitutively synthesized low levels of MMP-9 and huge amounts of its preferential inhibitor, tissue inhibitor of matrix metalloproteinase-1 (TIMP-1). In the present study, TIMP-1 phenotypic expression was extensively investigated in response to various mediators including interleukins, chemokines, growth factors and tumor promotor, and was compared to MMP-9 synthesis. Results showed a roughly constitutive TIMP-1 expression opposed to an inducible MMP-9 synthesis. Nevertheless, further analysis of TIMP-1 synthesis showed the existence of regulation mechanisms: modulation of intracellular Ca^{2+} concentration as well as cation ionophore monensin were demonstrated to influence TIMP-1 production and secretion. The precise pathways implicated in these regulation mechanisms are currently under survey. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: B lymphocyte; Epstein–Barr virus; Phenotypic regulation; TIMP; Matrix metalloproteinase; Ion movement

1. Introduction

The matrix metalloproteinases (MMPs) constitute a family of zinc-endoproteinases initially characterized by their ability to degrade the components of the extracellular matrix and basement membrane [1–3]. These enzymes are also involved in the release of cell surface receptors and signaling factors [4]. MMPs are, in general, rarely expressed in normal tissues, but are abundant in situations marked by rapid morphological changes, i.e. wound healing, embryogenesis and involution of reproductive tissues, and in pathological situations marked by tissue destruction such as inflammatory diseases and cancer. Their activity is controlled extracellularly by specific enzyme inhibitors, the tissue inhibitors of matrix metalloproteinase (TIMPs), of which four forms have been characterized [5–7]. However, in addition to blocking MMP activity, TIMPs have been shown to exert growth factor functions that are independent of their enzymatic inhibitory activity [8–11]. Moreover, TIMP-1, first identified as an erythroid potentiating factor [12,13], is also known to inhibit apoptosis in different cell lines [14,15] and to induce B cell differentiation [16,17].

Under physiological and some pathological conditions, MMP and TIMP expression is regulated by different effectors including growth factors, cytokines, steroid hormones and cellular oncogenes [18,19]. The response to these factors is not only MMP/TIMP-dependent, but also depends on the cell type studied.

B lymphocytes are immune cells whose main function is to produce antibodies in response to stimulation by foreign antigens. The activation process occurs within the context of lymphoid organs and requires a continuous recirculation of lymphocytes between blood and tissues [20–22]. Crossing basement membrane and migration through the stroma tissue requires specific tools able to degrade the extracellular matrix. B cells from different

Abbreviations: Con A, concanavalin A; EBV, Epstein–Barr virus; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; PMA, phorbol myristate acetate; Ret ac, *all trans*-retinoic acid; TIMP, tissue inhibitor of matrix metalloproteinase

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origins have been shown to synthesize MMP-2, MMP-9 and TIMP-1 [23,24].

B lymphocytes can be immortalized by Epstein–Barr virus (EBV) infection leading to the establishment of lymphoblastoid cell lines. EBV that infects the vast majority of humans is associated with an expanding number of neoplastic diseases including Burkitt's lymphoma and Hodgkin's disease, many of which progress to highly malignant lymphomas [25,26].

As MMPs and TIMPs are implicated in tumor invasion and metastasis, especially in Burkitt's lymphoma and neoplastic B cell lines [27,28], we chose to study expression of MMPs and TIMPs by EBV-immortalized B lymphocytes, which may be considered as premalignant cell lines. We have previously shown that EBV B cells are able to constitutively secrete MMP-9 (gelatinase B, 92-kDa type IV collagenase, EC 3.4.24.35) and its preferential inhibitor TIMP-1 [29]. Moreover, MMP-9 expression was demonstrated to be finely regulated by various physiological mediators. We examined here the regulation of TIMP-1 synthesis by EBV B lymphocytes and compared it to the MMP-9 expression pattern. Several types of mediators were chosen according to their involvement in B cell differentiation (interleukins IL-1β, IL-2, IL-6), in Burkitt's lymphomas (IL-1β, IL-6, IL-10) [30–32] or in EBV-induced tumorigenesis [33]. IL-10 is known to abolish angiogenesis and tumorigenicity of Burkitt's lymphoma cells [34], and retinoic acid is described as a powerful inhibitor of EBV B lymphocyte growth [35]. Other agents (TNFα, lipopolysaccharide (LPS), concanavalin A (Con A), etc.) are mediators of infectious or inflammatory situations. The results showed that TIMP-1 production is broadly constitutive whereas MMP-9 synthesis is inducible and highly controlled. Moreover, intracellular calcium is involved in the TIMP-1 basal and induced production, which suggested ion movements as a parameter of TIMP-1 regulation.

2. Materials and methods

2.1. Reagents

Reagents used in this work were obtained from the following sources: IL-10, LPS, Con A, phorbol myristate acetate (PMA), *all trans*-retinoic acid (Ret ac), monensin, ionomycin, BAPTA-AM (Sigma Chemical Co, St Louis, MO); IL-1β, IL-2, IL-6, TNFα, TGFβ (Roche–Boehringer Mannheim, Meylan, France).

2.2. Cell culture

Six different EBV B lymphocyte cell lines (B95-8 strain [36]) were cultured in an RPMI-1640 medium supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 50 μg/ml kanamycin and 50 μg/ml streptomycin (Gibco BRL, Life Technologies SARL, Cergy Pontoise, France). The culture was maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and the medium was changed twice weekly.

To study MMP and TIMP expression, FBS was removed from EBV B lymphocyte culture medium. Cells were washed three times in 40-ml FBS-free RPMI-1640 medium and then were grown for different culture times in the same medium containing 0.2% (w/v) BSA (Roche–Boehringer Mannheim) and in some experiments a stimulating agent. Cell viability was monitored by the trypan blue exclusion method; it was on the order of 90%.

2.3. RT-PCR analysis

Extraction of total RNA from control and stimulated EBV B lymphocytes by TRIzol™ Reagent (Gibco–Life Technologies) and reverse transcription to cDNA were performed as previously described [29]. A fragment of MMP-9 and actin cDNA (838 bp) or a portion of TIMP-1 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA (450 bp) were then amplified simultaneously in the same tube by PCR, using 2.5 U Taq polymerase (Appligene Oncor, Illkirch, France) in 100 μl Taq buffer containing 0.2 mM dNTP, 0.25 μM oligonucleotide primers for MMP-9 or TIMP-1 [29], and 0.125 μM commercial primers (Clontech Laboratories, Palo Alto, CA) for actin (sense 5′-294–325 bp/antisense 5′-1131–1100 bp) or G3PDH (sense 5′-586–605 bp/antisense 5′-1037–1018 bp). The PCR protocol was the same as used before [29]. Aliquots of 4 to 15 μl PCR products were run together with a scale of DNA ladders (markers VI, Roche–Boehringer Mannheim) on 1.5% (w/v) agarose gel containing 1 μg/ml ethidium bromide. Signal intensity of target (MMP-9 and TIMP-1) and internal control (actin and G3PDH) PCR products appearing on the gel photograph were digitalized by densitometry at 510 nm (CD 60, Desaga, Sarstedt Gruppe). Relative abundance was calculated as a ratio of MMP-9 to actin or TIMP-1 to G3PDH signals, as previously done [37].

PCR products were gel-purified and sequenced by Genome Express Ltd., Grenoble, France.

2.4. Northern blot analysis

Northern blot analysis was carried out using the previously published method [29]. Briefly, EBV B lymphocyte total RNA was extracted by the TRIzol™ technique and poly(A)$^+$ RNA was purified onto an oligo(dT) cellulose matrix. After denaturation at 65 °C for 15 min, mRNA was size-fractionated on a 1% (w/v) agarose–formaldehyde gel, blotted onto a positive nylon membrane by capillary action and immobilized by UV cross-linking. The membrane was then hybridized overnight at 42 °C with a PCR digoxigenin (DIG)-labeled probe of MMP-9 or TIMP-1 mRNA. After washing, the detection was performed using anti-digoxigenin (Fab) fragments conjugated to alkaline phosphatase followed by a chemiluminescent reaction (Roche–Boehringer Mannheim). Signals were detected by exposing the

blot onto Hyperfilm MP (Amersham Life Science, Buckinghamshire, UK). The membrane was then briefly washed and hybridized again with a DIG-labeled probe of G3PDH transcript. The mRNA integrity and the size of each characterized mRNA were assessed by visualizing the remaining 28S and 18S ribosomal RNA bands under UV light after staining the gel with ethidium bromide. Quantitation was performed by scanning densitometry at 400 nm of the MMP-9, TIMP-1 and G3PDH bands seen on X-ray films and was expressed as a ratio MMP-9 or TIMP-1 to G3PDH signals.

2.5. MMP-9 and TIMP-1 production by EBV B lymphocytes

MMP-9 and TIMP-1 were isolated from EBV B lymphocyte culture medium as previously published [29]. Briefly, washed cells were cultivated for 18 h in an FBS-free RPMI medium containing 0.2% BSA and a stimulating agent. The medium was then withdrawn and cells were further cultivated in the same medium for 72 h. MMP-9 was purified using substrate affinity chromatography on gelatin agarose (Sigma), characterized after Centricon 10 concentration onto a 0.05% (w/v) gelatin zymography and quantitated by scanning densitometry and reference to a purified MMP-9 standard curve. Free TIMP-1 and MMP-9 complexed TIMP-1 were measured in the culture medium using a commercial ELISA assay (Amersham Life Science). TIMP-1 was also investigated inside the cells: EBV B lymphocytes were sonicated three times for 10 s at 40 W, the homogenate was centrifuged at $500 \times g$ for 10 min and TIMP-1 was quantitated by ELISA in the supernatant. All the experiments were standardized by using an equal number of cells and P values were calculated by the Student's paired t test.

2.6. Flow cytometry

EBV B lymphocytes stimulated by 10 ng/ml LPS were cultured for 18 h with or without (control) 2.5 μM monensin and were suspended in PBS at a 3×10^6 cells/ml concentration. Cells were fixed with 3% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde and were resuspended in 1 ml PBS containing 1% (w/v) BSA, 0.001% saponin and 10% (v/v) rabbit nonimmune serum, which had been previously heated for 30 min at 63 °C to reduce nonspecific binding of antibodies. They were then incubated at 4 °C for 45 min with rabbit polyclonal Ig anti-rTIMP-1 (1:20), generated with rTIMP-1 (a generous gift from Synergen). After two washes, a solution of FITC-conjugated goat anti-rabbit IgG (1:1000) (Immunotech, Marseille, France) was added to the lymphocytes and after 45 min at 4 °C, cells were used for flow cytometry. Samples were analyzed on a FACScan (Becton Dickinson) fitted with an argon ion-laser at 488 nm. Fluorescence intensity was measured on a logarithmic scale and the mean fluorescence of the analyzed cells was calculated using FACScan research software (Becton Dickinson).

2.7. Intracellular calcium measurement

This method was developed from a previously described technique [38]. EBV B lymphocytes were incubated for 15 min at 37 °C with 2.5 mM probenecid (Sigma) and 1 mM fura-2 AM (Sigma) in an RPMI-1640 medium without phenol red (Gibco–Life Technologies) or any complements and then resuspended at a 4×10^6 cells/ml concentration in the same medium. The fluorescence of the fura-2 AM (excitation wavelength 340 nm, emission wavelength 505 nm) was measured for 10^7 cells at 37 °C under slow magnetic agitation during the entire experiment. After reaching a plateau, 1 ml of 100 mM $MnCl_2$ was added to the curve to chelate extracellular fura-2 AM. After 10 s, 2 ml of diethylenetriaminepentaacetic acid (DTPA) (Sigma) was added to chelate $MnCl_2$. Cells were then lysated by 50 μl of 20% (v/v) Triton X-100. Fura-2 AM nonligated calcium was chelated by 25 μl of 0.5 M EGTA and the pH was finally neutralized with 25 μl of 1 M Tris. Intracellular calcium concentration was determined using the

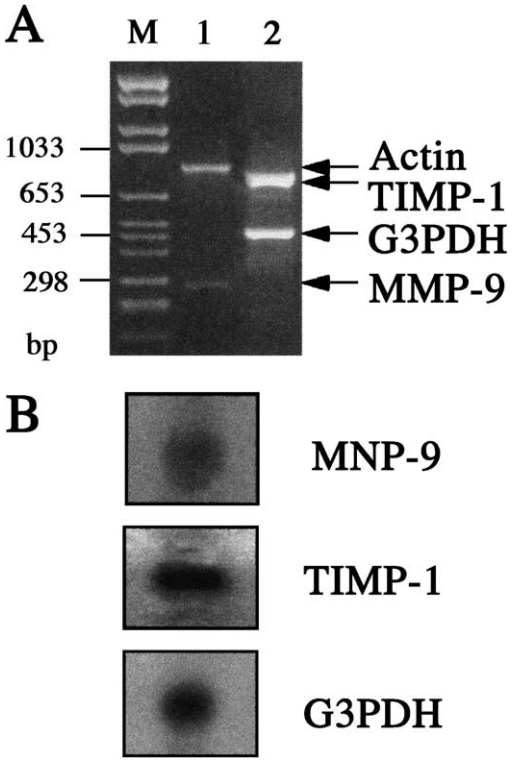

Fig. 1. TIMP-1 and MMP-9 mRNA characterization by RT-PCR (A) and by Northern blot (B) analysis. Total RNA was extracted from unstimulated EBV B lymphocytes cultivated in an RMPI medium containing 10% FBS. (A) mRNA was reverse transcribed using an oligo(dT) primer and two double PCRs were performed. PCRs with primers for MMP-9 and actin (lane 1) or TIMP-1 and G3PDH (lane 2) yielded amplification products of the expected sizes of 277 bp (MMP-9) and 838 bp (actin), or 769 bp (TIMP-1) and 450 bp (G3PDH). (B) mRNA was purified onto an oligo(dT) cellulose matrix, then loaded on an agarose–formaldehyde gel and blotted onto a nylon membrane. Total mRNA of MMP-9, TIMP-1 and G3PDH were characterized with specific digoxigenin-labeled probes. The figure is a representative example of at least 10 experiments.

Table 1
Regulation of TIMP-1 transcription and protein synthesis in EBV B lymphocytes

Stimulating agent[a]	RT-PCR		Northern blot	Proteins	
	8 h	14 h	24 h	18 h	72 h
Control	100 ± 1 (9)	100 ± 2 (8)	100 (4)	100 ± 73 (6)	100 ± 32 (6)
IL-1β[b]	105 ± 2 (3)	91 ± 3 (3)	ND[c]	66 ± 15 (4)	131 ± 37 (5)
IL-2	102 ± 2 (3)	92 ± 7 (3)	ND	163 ± 37 (4)	138 ± 34 (4)
IL-6	97 ± 5 (3)	97 ± 2 (3)	ND	143 ± 33 (4)	164 ± 38 (4)
IL-10	110 ± 2 (3)	117 ± 2 (3)	ND	122 ± 22 (4)	179 ± 42 (4)*
TNFα	110 ± 3 (3)	77 ± 0 (3)	ND	322 ± 89 (4)	219 ± 50 (4)
TGFβ	93 ± 2 (3)	79 ± 3 (3)	151 ± 91 (5)	336 ± 40 (3)*	225 ± 53 (3)*
LPS	85 ± 6 (4)	85 ± 4 (4)	107 ± 24 (3)	709 ± 138 (9)*	292 ± 52 (5)*
Con A	80 ± 2 (3)	77 ± 2 (3)	ND	125 ± 44 (5)	35 ± 9 (3)
PMA	73 ± 1 (3)	72 ± 1 (3)	97 ± 8 (3)	859 ± 258 (5)*	525 ± 122 (4)*
Ret ac	74 ± 2 (3)	71 ± 1 (3)	332 ± 68 (4)*,[d]	309 ± 107 (5)	335 ± 77 (5)*

[a] Concentration used are 1 ng/ml IL-1β, 25 ng/ml IL-2, 10 ng/ml IL-6, 20 ng/ml IL-10, 20 ng/ml TNFα, 1 ng/ml TGFβ, 10 ng/ml LPS, 1 μg/ml Con A, 0.5 nM PMA and 10 μM retinoic acid.
[b] Each value was reported to the value 100 of unstimulated cells (control) and represents a mean ± S.E. (number of experiments).
[c] ND: not determined.
[d] *P* values were calculated by Student's paired *t* test.
* $P<0.05$ (stimulation versus control).

fura-2 AM Kd (224 nM), the baseline fluorescence and the fluorescence after cell lysis and calcium chelation by EGTA.

3. Results

3.1. Expression of TIMP-1 and MMP-9 transcripts by EBV B lymphocytes

The capacity of EBV B lymphocytes to express TIMP-1 and MMP-9 mRNA was studied using RT-PCR and Northern blot. Firstly, total RNA was reverse transcribed to cDNA. Then a 769-bp portion of TIMP-1 transcript and a 277-bp fragment of MMP-9 cDNA were amplified by the Taq polymerase (Fig. 1A). G3PDH or actin cDNA were used as internal controls. The sequencing of each PCR product showed, respectively, 99.3% (TIMP-1) and 99.5% (MMP-9) homology with the published sequences [39,40]. No PCR products were characterized without RT (data not shown).

The entire mRNA of TIMP-1 and MMP-9 was also characterized by Northern blot analysis, using digoxigenin-labeled probes (Fig. 1B). The size of each mRNA was estimated using the 18S and 28S ribosomal RNA. Both mRNAs were isolated at the size previously published [39,40], i.e. approximately 810 bp for the TIMP-1 mRNA and 2300 bp for the MMP-9 transcript. A second hybridization of the positive nylon membrane was then performed with a G3PDH cDNA probe.

Quantification of PCR products and Northern blot-characterized total mRNA of TIMP-1 and MMP-9 was performed by densitometric scanning of each signal. Results

Table 2
Regulation of MMP-9 transcription and protein synthesis in EBV B lymphocytes

Stimulating agent[a]	RT-PCR		Northern blot	Proteins	
	8 h	14 h	24 h	18 h	72 h
Control	100 (15)	100 (10)	100 (10)	100 ± 6 (10)	100 ± 16 (5)
IL-1β[b]	203 ± 23 (4)*,[c]	ND	ND	87 ± 9 (3)	239 ± 47 (5)*
IL-2	270 ± 9 (3)*	214 ± 24 (6)*	ND	27 ± 13 (3)*	56 ± 9 (5)
IL-6	ND[d]	75 ± 4 (5)	ND	47 ± 23 (3)*	50 ± 12 (4)
IL-10	154 ± 5 (3)*	ND	ND	67 ± 13 (4)*	100 ± 22 (4)
TNFα	ND	162 ± 11 (9)*	ND	27 ± 14 (3)	22 ± 7 (5)*
TGFβ	142 ± 12 (5)*	103 ± 7 (9)	149 ± 47 (7)*	0 (3)*	0 (3)*
LPS	382 ± 13 (5)*	181 ± 13 (5)*	213 ± 39 (7)*	393 ± 24 (6)*	244 ± 43 (6)
Con A	272 ± 7 (4)*	151 ± 18 (4)*	228 ± 29 (7)*	507 ± 67 (3)*	300 ± 62 (4)
PMA	273 ± 12 (4)*	113 ± 8 (4)*	134 ± 17 (8)	234 ± 29 (12)*	ND
Ret ac	379 ± 16 (4)*	254 ± 22 (4)*	205 ± 30 (5)*	173 ± 37 (3)	ND

[a] Concentrations used are detailed in Table 1.
[b] Each value was reported to the value 100 of unstimulated cells (control) and represents a mean ± S.E. (number of experiments).
[c] *P* values were calculated by Student's paired *t* test.
[d] ND: not determined.
* $P<0.05$ (stimulation versus control).

are expressed as a ratio TIMP-1 or MMP-9 to G3PDH or actin, which allows estimation of the abundance of TIMP-1 and MMP-9 mRNA in response to the different agents (Tables 1 and 2).

3.2. Regulation of TIMP-1 versus MMP-9 expression by EBV B lymphocytes

TIMP-1 and MMP-9 synthesis by EBV B cells was investigated at two levels: firstly at the transcription level, by scanning PCR products and Northern blot-characterized mRNA, and then at the protein level. TIMP-1 was quantified by ELISA and MMP-9 was analyzed by zymography followed by a densitometric quantification [29]. We analyzed mRNA after 8 and 14 h for RT-PCR experiments and 24 h for Northern blot analysis. Proteins were measured in an 18-h FBS-free medium containing a stimulating agent and a continuing 72-h identical medium. The optimal dose of most agents has been previously determined by studying the effect of at least three concentrations (data not shown). Results are reported in Tables 1 and 2 and expressed as the percentage of the control values (mRNA or protein).

Concerning TIMP-1, the results of all PCR reactions showed total independence of mRNA expression whatever stimulating agent was used. Only retinoic acid significantly induced the amount of TIMP-1 transcript, as determined by Northern blot (Table 1).

In contrast to TIMP-1, the majority of the tested mediators significantly induced the MMP-9 transcript synthesis (Table 2). IL-1β, IL-2, IL-10, TNFα, LPS, Con A, PMA and retinoic acid increased the amount of mRNA after 8 and/or 14 h of culture, whereas the stimulation effect of TGFβ decreased after 14 h of culture. The significant induction of MMP-9 mRNA by TGFβ, LPS, Con A and retinoic acid was confirmed by Northern blot analysis (Table 2).

The study on TIMP-1 and MMP-9 phenotypic expression investigated not only mRNA transcription but also protein synthesis. Under unstimulated conditions, TIMP-1 production by 10^7 EBV B lymphocytes fluctuated between 2 and 21 ng depending on the EBV cell line after 18 h of culture in an FBS-free medium. About 0.15 ng of MMP-9 was produced by these cells during the same period. After another 72 h of culture, TIMP-1 production was close to 38 ng, whereas MMP-9 synthesis increased to 0.18 ng. We had previously shown that all MMP-9 produced was secreted outside of the cells [29].

As transcript synthesis, TIMP-1 protein expression is barely influenced by extracellular mediators. Nevertheless, some ligands were shown to strongly up-regulate the inhibitor synthesis (Table 1). TGFβ, LPS and PMA increased TIMP-1 production after 18 h of culture and an additional 72 h of incubation. The increased TIMP-1 synthesis observed with retinoic acid and IL-10 was only significant after 90 h of culture with these mediators. No other agents induced any significant effect on TIMP-1 expression.

Concerning MMP-9, basal expression was affected by exposure of EBV B lymphocytes to different mediators (Table 2). Increased levels of secreted MMP-9 were observed after 18 h of culture of the cells with PMA, LPS and Con A: MMP-9 basal production was increased twofold, threefold and fivefold, respectively, in agreement with the MMP-9 mRNA elevated level previously measured (multiplicative factor of 2.7, 3.8 and 2.7 determined by semi-quantitative RT-PCR). MMP-9 expression was also increased by IL-1β but required at least 30 h to develop [29]. On the other hand, the enzyme production was decreased by IL-2, IL-6, IL-10 and TNFα and was totally abolished by TGFβ. Finally, retinoic acid failed to signifi-

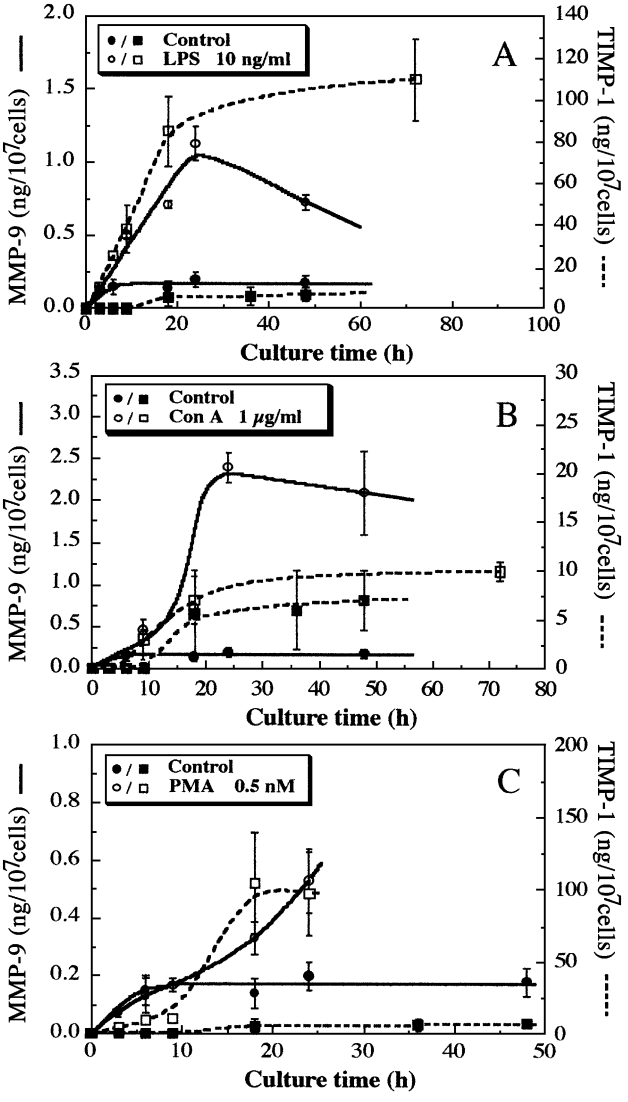

Fig. 2. Kinetics of MMP-9 and TIMP-1 synthesis by EBV B lymphocytes in the presence of LPS (A), Con A (B) and PMA (C). Cells were cultivated in an FBS-free RPMI medium containing 0.2% BSA and 10 ng/ml LPS (A), 1 μg/ml Con A (B) or 0.5 nM PMA (C). Medium was withdrawn after different culture times; TIMP-1 was quantified by ELISA and MMP-9 was analyzed by zymography and quantitated by scanning densitometry.

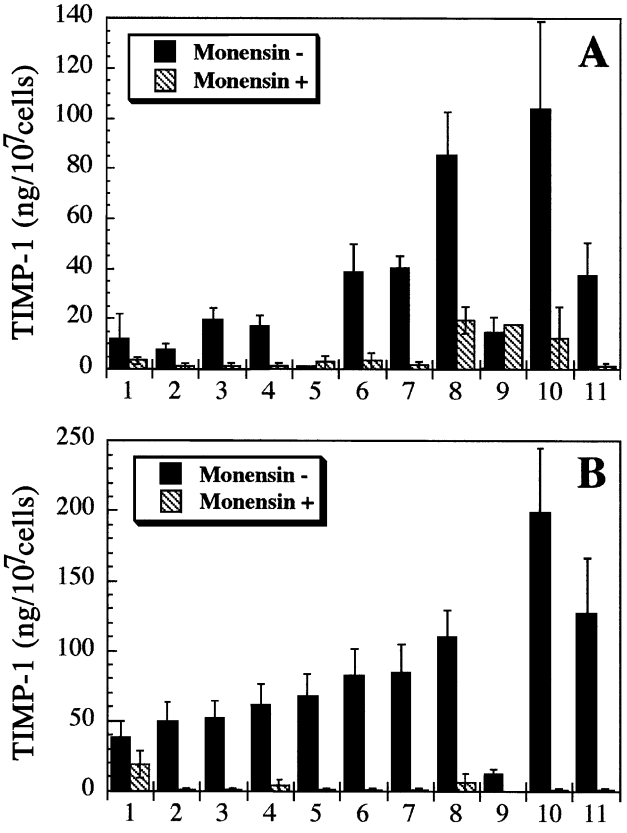

Fig. 3. Quantitative analysis of TIMP-1 secretion by EBV B lymphocytes after 18 h (A) or 72 h (B) of culture. Cells were cultivated for 18 h in the presence of a stimulative agent with (hatched) or without (black) monensin. The medium was then withdrawn and cells were further cultured in the same conditions for 72 h. TIMP-1 was quantified by ELISA in both media. The stimulative agents used were 1 ng/ml IL-1β (lane 2), 25 ng/ml IL-2 (lane 3), 10 ng/ml IL-6 (lane 4), 20 ng/ml IL-10 (lane 5), 20 ng/ml TNFα (lane 6), 1 ng/ml TGFβ (lane 7), 10 ng/ml LPS (lane 8), 1 μg/ml Con A (lane 9), 0.5 nM PMA (lane 10) and 10 μM retinoic acid (lane 11). Basal TIMP-1 production from unstimulated EBV B lymphocytes is shown in lane 1. The error bars represent the S.E. of at least three experiments.

cantly up- or down-regulate MMP-9 production, whereas it stimulated mRNA synthesis. The discrepancies between mRNA abundance and protein level suggest a posttranscriptional regulation of MMP-9 expression.

Among all cytokines, growth factors and other tested mediators, LPS, Con A and PMA were the three agents that exerted the strongest stimulation effect on TIMP-1 and/or MMP-9 synthesis. The production kinetics of TIMP-1 and MMP-9 in response to these agents was then examined (Fig. 2). TIMP-1 synthesis differed depending on the stimulating ligand: the inhibitor production was dramatically increased by LPS and PMA during the first hours of culture until the production reached a plateau, whereas Con A did not exert any significant stimulative effect on this expression, as was previously shown (Table 1). Concomitantly LPS, Con A and PMA strongly induced MMP-9 synthesis (respectively, 7-, 15- and 3-fold) during the first 24 h of culture and then the enzyme level slowly decreased.

3.3. Effect of monensin on TIMP-1 production and secretion

The expression and secretion mechanisms of TIMP-1 were then investigated using the carboxylic ionophore monensin. This compound has been reported to alter protein secretion by its ability to collapse Na^+ and H^+ gradients across intracellular membranes [41]. EBV B lymphocytes were incubated with the different stimulating agents and 2.5 μM monensin for 18 h (Fig. 3A) and then for an additional 72 h (Fig. 3B); secreted TIMP-1 was measured in the extracellular medium using ELISA. Treating the cells with the ionophore strongly decreased and sometimes abolished TIMP-1 secretion, whatever the stimulating agent and the culture time.

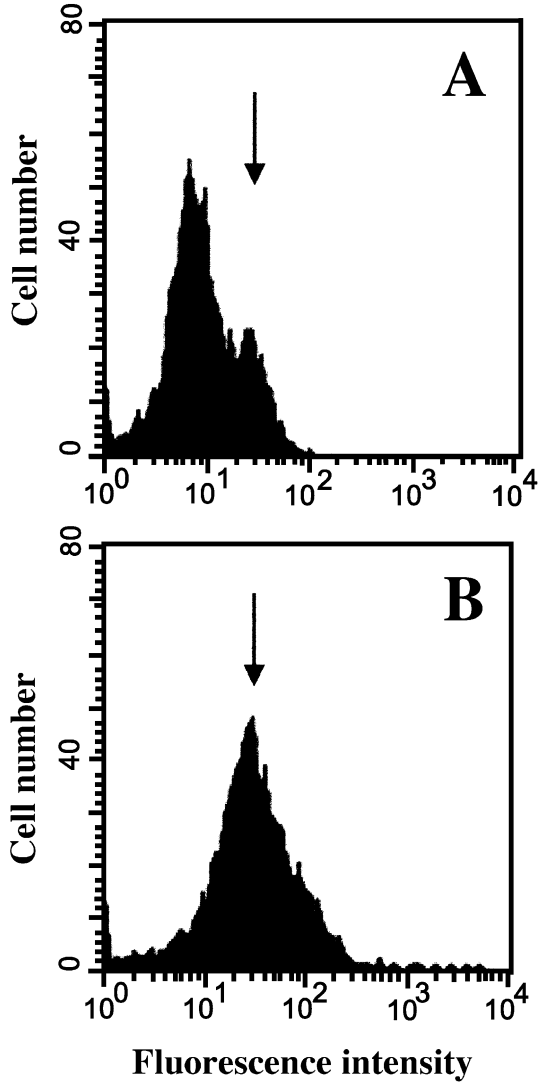

Fig. 4. Immunolocalization of TIMP-1 in EBV B lymphocytes. LPS-stimulated cells (3×10^6) incubated for 18 h with (B) or without (A) 2.5 μM monensin were suspended in PBS. They were then fixed, permeabilized and incubated with rabbit anti-TIMP-1 Ig and then with FITC-conjugated goat anti-rabbit IgG for flow cytometry. The arrow illustrates the fluorescent shift and the cellular localization of TIMP-1 upon monensin incubation.

To investigate the monensin effect on TIMP-1 secretion, EBV B lymphocytes were fixed after incubation with LPS alone (control) and monensin for flow cytometry analysis. The presence of monensin induced a shift in the main fluorescence peak corresponding to the intracellular TIMP-1 (arrow) (Fig. 4B), which increased from 11.5 to 41.7, illustrating that in the presence of monensin, TIMP-1 was not secreted in the medium and accumulated inside the cells.

Nevertheless, it has been previously shown that monensin is able to reduce not only protein secretion but also protein synthesis by 20% in some in vitro culture models [42]. The intra- and extracellular amount of TIMP-1 secreted by EBV B lymphocytes in response to monensin was then quantitated by ELISA (Fig. 5). Without monensin, as previously shown, TIMP-1 was broadly secreted outside of the cells whether they were unstimulated or cultivated with LPS. When monensin was added to the culture medium, TIMP-1 secretion by LPS-stimulated EBV B lymphocytes was decreased by 80% (Fig. 5, Table 3). Concurrently, the amount of intracellular TIMP-1 in monensin-treated cells increased 2.5-fold. Nevertheless, TIMP-1 total production by 10^7 monensin-cultivated EBV B lymphocytes was about 30 ng, whereas untreated cells synthesized and released about 90 ng TIMP-1; this corresponds to a 66% decrease in TIMP-1 synthesis by monensin-cultured EBV B cells.

3.4. Importance of calcium in the control process of TIMP-1 synthesis

The role of Ca^{2+} in TIMP-1 production was investigated using the specific Ca^{2+} chelator BAPTA. BAPTA-AM, a membrane-permeable form of BAPTA, is able to penetrate a wide variety of cells without affecting their viability and is hydrolyzed intracellularly by cytosolic esterases to become active [43]. The intracellular Ca^{2+} concentration of control or LPS-stimulated EBV B lymphocytes ranged from 155 to 180 nM (Table 3). When unstimulated cells were incubated with 20 μM BAPTA-AM for 18 h, the Ca^{2+} concentration was reduced to an undetectable level. The simultaneous measurement of TIMP-1 in the extracellular medium showed a decreased secretion of 43% (Table 3). No TIMP-1 accumulation was found inside the cells (data not shown), suggesting that BAPTA had an effect on protein synthesis. In the presence of the carboxylic ionophore ionomycin, which allows calcium influx, 91% basal intracellular Ca^{2+} concentration and 96% TIMP-1 production, as measured in the extracellular medium, were recovered. These results suggest that the decrease in TIMP-1 production by BAPTA-AM-cultivated cells was due to an alteration of intracellular Ca^{2+} concentration.

Moreover, no modification of cytoplasmic Ca^{2+} level was observed in the presence of 2.5 μM monensin, whereas TIMP-1 concentration strongly decreased compared to the LPS control level, as previously shown (Fig. 5): the monensin-induced decrease of extracellular TIMP-1 was independent of cytoplasmic Ca^{2+} concentration.

Table 3
Influence of Ca^{2+} concentration on TIMP-1 production by EBV B lymphocytes

Culture conditions	Extracellular TIMP-1 (% control)	Intracellular $[Ca^{2+}]$ (nM)
Control[a]	100 ± 73	180 ± 24
LPS (10 ng/ml)	709 ± 138	155 ± 21
LPS + Mon[b]	147 ± 62	183 ± 20
BAPTA (20 μM)	57 ± 3	undetected
BAPTA + Iono[b]	96 ± 28	152 ± 12

[a] Each value represents a mean ± SE of at least duplicate determinations.
[b] Monensin (Mon) was used at a 2.5 μM concentration and ionomycine (Iono) at a concentration of 5 μg/10^6 cells.

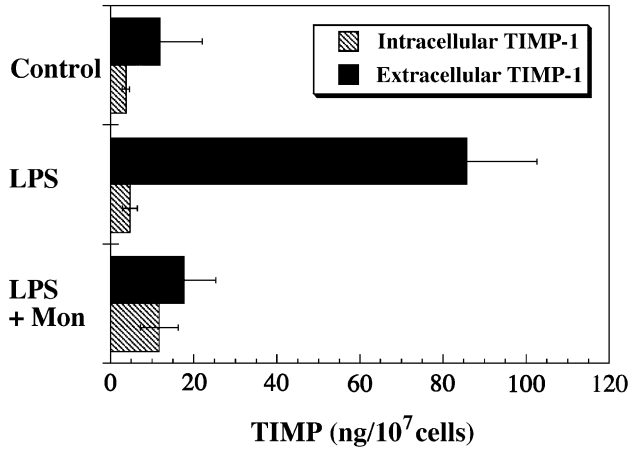

Fig. 5. Influence of monensin on TIMP-1 synthesis by EBV B lymphocytes. Unstimulated and LPS-stimulated cells, with or without 2.5 μM monensin, were cultivated for 18 h. The medium was then withdrawn and cells were sonicated. TIMP-1 was quantified by ELISA in the extracellular medium and in the cell lysate. The error bars represent the S.E. of at least three experiments.

4. Discussion

The main aim of this study was to describe TIMP-1 regulation mechanisms in EBV B lymphocytes. We first demonstrated the roughly constitutive expression of the inhibitor and we then characterized the significant effect of monensin on TIMP-1 production and secretion. The results showed the direct involvement of Ca^{2+} in TIMP-1 basal synthesis by EBV B cells.

Indeed, TIMP-1, first described as an erythroid potentiating factor [12], has also been described as having growth factor [10] and anti-angiogenic activities [44], and more recently as acting as an anti-apoptotic factor [14,15] and a modulator of B cell differentiation [16,17]. Moreover, its expression has been correlated with differential prognosis in cancer according to the neoplasm type [45–49]. In particular, its expression and role was studied in Burkitt's lym-

phoma, showing an initial growth factor effect on the tumor followed by an angiogenic inhibition period [50,51]. Understanding TIMP-1 regulation would allow better control of these different activities.

TIMP-1 expression by EBV B lymphocytes was examined in response to different kinds of mediators that influence B lymphocyte phenotype (IL-1β, IL-2, IL-6, IL-10, etc.), EBV B cell proliferation (retinoic acid) [35] or lymphocyte response in pathological situations (TNFα, LPS, Con A, etc.). IL-6 also plays a role in EBV-induced B cell tumorigenesis [33] and IL-10 has anti-angiogenic and anti-tumorigenic activities on Burkitt's lymphoma cells [34]. MMP-9 synthesis was also investigated at the same time and compared to TIMP-1 production. Enzyme transcript and protein synthesis were shown to be finely regulated: LPS, Con A and PMA greatly increased MMP-9 production, whereas other mediators such as IL-2, IL-6, TNFα and TGFβ partially inhibited or totally abolished the enzyme synthesis. Nevertheless, MMP-9 synthesis was always much lower than TIMP-1 production whatever stimulating mediator was used, which results in a great MMP-9/TIMP-1 imbalance in favor of the inhibitor. We looked for 14 other MMPs and TIMPs by RT-PCR; only MMP-7 (matrilysin) and MMP-16 (membrane type 3 MMP) transcripts were isolated and both proteins were characterized at a very low level (data not shown). All these results suggested a function for EBV B cell TIMP-1 other than an MMP inhibitor, as was discussed previously [14,24].

As in T lymphocytes [52] and macrophages [53], TIMP-1 production by EBV B cells is broadly constitutive. We found that mRNA synthesis was unchanged whatever the stimulating agent used, and protein synthesis was only enhanced by a few specific ligands: only IL-10, TGFβ, LPS, PMA and retinoic acid were able to induce TIMP-1 production, as previously described from different sources [19]. Nevertheless, the response delay to IL-10 and retinoic acid might result from several gene-expression cascades.

IL-10 was previously shown to greatly increase TIMP-1 synthesis in native B lymphocytes whose basal production is much lower than in immortalized cells [24]. Moreover, TIMP-1 synthesis and secretion have also been up-regulated by this cytokine in other cell types, suggesting an induction process via an autocrine mechanism [54,55]. Recent data characterizing a TIMP-1 induction of IL-10 in Burkitt's lymphoma cell lines confirmed this hypothesis [17]. Several in vitro studies reported a correlation of TIMP-1 and IL-10 elevated levels with the high grade of tumors and the poor outcome of patients [16,17], but both proteins were also shown to alter tumorigenicity and angiogenesis of Burkitt's lymphoma in vitro or in mouse models [34,51]. Prognosis significance of TIMP-1 and IL-10 needs to be clarified by clinical studies.

Calcium is known not only to be necessary to metalloproteinase activity [1], but also to modulate MMP expression and/or activation [56–59]. Moreover, synthetic calcium influx inhibitors were shown to inhibit cancer cell growth and invasion in vitro [60] and in vivo [61], acting through a strong decrease in MMP synthesis. The importance of Ca^{2+} as a second messenger in signaling pathways was also described for TIMP-1 induction in Sertoli cells [62]. We have reported here, for the first time, that Ca^{2+} is directly involved in TIMP-1 basal synthesis in EBV B lymphocytes. The intracellular Ca^{2+} chelator BAPTA greatly decreased TIMP-1 production, which is restored by the Ca^{2+} ionophore ionomycin concomitantly with calcium concentration.

TIMP-1 synthesis and secretion were also shown to be strongly decreased by monensin, a carboxylic ionophore inhibiting protein extracellular secretion. Basal and induced productions were affected, which could be explained by a slow-down of the autocrine loop due to the global decrease in TIMP-1 synthesis. Down-regulation of TIMP-1 production was associated with an accumulation inside the cells, as was previously described in human endometrium treated with monensin [63]. Moreover, monensin, which is also known to raise cytoplasmic calcium in some bovine cells [64], has no influence on B lymphocyte intracellular Ca^{2+} and thus acts at a different level. These two experiments, both showing a partial inhibition of TIMP-1 synthesis, brought out the existence of several independent pathways leading to the inhibitor production.

Our study was done on EBV-immortalized B lymphocytes. As disregulation of metalloproteinases and TIMPs synthesis is implicated in tumor invasion process and metastasis, knowledge of MMP and TIMP expression and regulation by premalignant lymphoblastoid cell lines is very relevant. Indeed, in this paper we have shown a broadly constitutive high expression of TIMP-1 by EBV B cells and an inducible weak synthesis of MMP-9. These results contrast with MMP-9/TIMP-1 expression in normal B cells whose TIMP-1 synthesis is much lower than MMP-9 production [24]. Synthesis of a huge amount of TIMP-1 versus the MMP-9 low production argues in favor of the preponderant role of the inhibitor in EBV B lymphocyte physiology. The interesting new findings reported here are the importance of calcium ions on basal and induced TIMP-1 production as well as the existence of an autocrine regulation mechanism. These precise pathways of TIMP-1 synthesis are currently under investigation.

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